2016

The Effects of Tillage, Glyphosate, and Genetic Modification on Bacterial Root Endophyte Composition in Zea Mays

Breena Lori Nolan
University of Mississippi. Sally McDonnell Barksdale Honors College

THE EFFECTS OF TILLAGE, GLYPHOSATE, AND GENETIC MODIFICATION ON BACTERIAL ROOT ENDOPHYTE COMPOSITION IN *ZEA MAYS*

by

Breena Lori Frieda Nolan

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford

November 2016

Approved by

_____

Advisor: Dr. Colin Jackson

_____

Reader: Dr. Michael Jenkins

_____

Reader: Dr. John Samonds

ACKNOWLEDGEMENTS

Many thanks to USDA-ARS for supplying the experimental design, technical knowledge, and materials for this experiment, as well as preparing the root material for processing, particularly Dr. Michael Jenkins and Dan McChesney. Thanks as well for the financial support for this experiment provided by the Sally Barksdale Honors College and USDA-ARS. Finally, many thanks to Dr. Colin Jackson, who refined the experimental design, provided all training with mothur and the associated programs, and advised throughout the production of this thesis. It is thanks to their efforts that this paper was written.

ABSTRACT

The community structure of the endophytic bacteria in *Zea mays* roots was examined for the potential effects of glyphosate application, tillage strategies, and whether or not the corn plant in question was of an organic or glyphosate-resistant variety. Roots were harvested from plots designated to receive their specific treatments at the USDA-ARS Crop Production Systems Research Unit Farm. Vortexing, sonication, and tissue grinding, extraction, and next generation sequencing of 16S rRNA genes from these roots were used to describe their bacterial community composition. Results indicated significant differences in the bacterial communities correlated to tillage practice or corn type, whereas glyphosate treatments did not seem to affect the bacterial community. There also appeared to be certain holistic differences resulting from the combinations of certain treatments. Prior research has focused primarily on fungal endophytes, but as 16S rRNA sequencing has immeasurably broadened the scope of microbiological studies, new research such as this seeks to identify new microbes and their potential functions in the macroscopic world.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Over the last few decades, advances in DNA sequencing technology have led to previously unexplored avenues of research in microbiology. Many microorganisms were impossible to identify prior to 16S rRNA and rDNA analysis methods [13], resulting in extensive gaps in our knowledge of the diversity studies of virtually all microbial communities. Between 2001 and 2007, the use of 16S rDNA sequencing identified 215 new bacterial species from human specimens alone [18]. Although the library of known rRNA sequences is far from complete, 10^6 rRNA sequences have been identified as of 2010, providing for 10^9 distinct 16S rRNA gene sequence tags [13], which allow the classification and study of novel microbes that have never been cultured, are rare, or are particularly slow-growing. Once an unknown microorganism has been isolated, today's molecular phylogeny techniques can be used to determine its relatives in an effort to better understand these new specimens' function in the microbial community [14]. Sequencing techniques themselves have also been improving rapidly. The development of pyrosequencing has allowed for the identification of nearly 100-fold more sequences than the traditional Sanger method [12]. The Illumina HiSeq and MiSeq platforms offer affordable, high-throughput sequencing, greatly expanding the range of feasible microbe studies and experiments [2].

Among these newer areas of study are those that focus on niches that were previously difficult to access without disrupting or killing the bacteria in question; for instance, the internal tissues of plants. These limitations are in part responsible for the classic definition of an endophyte as "fungi colonizing living plant tissue without causing any immediate, overt negative effects" [6]. This overlooks the ubiquitous prevalence of bacterial endophytes in every plant,

and furthermore disregards that these symbiotic relationships may be a major component of that plant's growth and survival [17]. Endophytes encompass the full spectrum of symbiotic interactions, with the only true defining feature of this group being the ability to live inside the plant host's tissue without killing its host [17]. As a reservoir of genetic diversity that likely contains many undiscovered species, bacterial endophytes may offer extensive insight into microbial diversity and phylogeny [17]. Model research systems of endophytes may also lead to a broader understanding of plant-pathogen interactions and evolution.

Interest in prokaryotic endophytes has largely developed in agriculture, where interactions between endophytes, the plant, and the broader environment have economic impacts. For example, the nitrogen-fixing bacterium *Acetobacter diazotrophicus*, an endophyte of sugarcane, allows the crop to be grown for long periods of time without the need to replenish soil nitrogen through fertilizers [1]. This suggests that an improved understanding of how human cultivation practices impact the microbial community and vice versa may in turn lead to higher crop yields, hardier plants, and reduced capital lost on fertilization and pesticides. Likewise, parasitic and disease-causing microorganisms might be easier to combat once a plant species' internal microbial community is better understood.

A cultivation practice that is currently being examined is soil tillage, which can affect the soil microbial community and lead to changes in nitrogen, phosphorus, and carbon cycling. In turn, these can result in variations in plant growth, as well as affect the competitive inhibition of plant pathogens. Reduced tillage, as opposed to conventional tillage, typically increases the amount of carbon in the soil and total bacterial biomass in upper soil horizons, without affecting bacterial growth rate [3, 5]. However, prior tillage studies have never taken into account the

specific bacterial community composition, nor have they considered the endophytes in the crops grown in such systems.

As well as tillage practices, effects of pesticides on soil and endophyte bacterial communities are also of interest, particularly the effects of glyphosate, N-(phosphonomethyl)glycine, the active ingredient in Roundup herbicide [9]. Roundup is a post-emergence, non-selective herbicide that works by inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase in the shikimate pathway [4], which would normally create aromatic amino acids essential to protein and secondary metabolite synthesis in plants [9], leading to a quick death upon exposure. A high uptake rate in plants, little degradation by plant metabolism, low mobility in soil and groundwater, as well as being reportedly relatively non-toxic to animals and non-carcinogenic [4], are traits that have made the use of Roundup widespread. However, glyphosate causes enzymatic and reproductive disruptions in animals, including a study that showed human placental cells begin to sustain damage at Roundup concentrations 10 times lower than those used in agriculture, with the effect increasing over time [11]. Roundup also directly inhibits aromatase activity in human microsomes, though pure glyphosate had a much lessened effect as opposed to Roundup itself [11]. Glyphosate's major degradation product, aminophosphonic acid is much more mobile in the soil and might also have an impact on microbial communities [4].

Another cultivation practice that relates to herbicide use is the increased production of genetically modified plants. One example, glyphosate resistance, is seen in transgenic glyphosate-resistant (GR) soybeans, which, when healthy, are able to metabolize glyphosate by means of glyphosate oxidoreductase (GOX). Resistance can also be developed by insertion of the CP4 gene of *Agrobacterium* into the desired plant genome [4], causing the production of GR

5-enolpyruvyl-shikimate-3-phosphate synthase. Knowing how the bacterial endophytes of GR plants compare to those within their unmodified originals could provide information on the broader effects of genetically modified crops.

Tillage, glyphosate application, and GR plants are interrelated practices. Glyphosate treatments allow reduced tillage more easily by controlling weeds, and this tillage practice is considered desirable due to its preservation of the top soil, prevention of the pollution of surface waters and air, and the indirect reduction of carbon dioxide emissions [4]. However, to use glyphosate, GR crops are required. Knowing how each of these cultivation practices affects the endophytic community both individually and in conjunction may prove to be of incalculable benefit to the agricultural community.

The aim of this research was to provide an initial analysis of the endophytes found in *Zea mays* roots, with a particular emphasis on whether endophyte composition would be affected by tillage methods (conventional versus reduced), pesticide exposure (glyphosate treatment versus control), and genetic modification (GR plants versus control plants). This research was part of a broader ARS-USDA study examining these treatments in a larger environmental context. Genetic material was extracted from *Zea mays* root samples and portions of bacterial 16S rRNA were examined using next generation sequencing. Results suggested only minimal influence of glyphosate upon the microbial community, while tillage method and genetic modification appeared to substantially alter the bacterial endophyte community composition.

METHODS

Study Site and Treatments

All samples were collected in 2014 from the USDA-ARS Crop Production Systems Research Unit Farm near Stoneville, MS. Plots consisted of eight 32m long rows spaced 1m apart. Three variables were tested with four replications: tillage (conventional versus reduced), glyphosate (treatment versus no exposure), and *Zea mays* genetic modification (transgenic GR plants versus non-GR). Field preparation was carried out via disking, subsoiling, disking, and bedding in 2014. Conventionally tilled plots were subsoiled and prepared following a corn harvest each fall, while reduced tillage plots were not tilled after the fall of 2014. Weeds were eliminated either by herbicide application or hand hoeing. Herbicide application followed a strict routine for every plot throughout the duration of the experiment: in February, all plots were burned down with 2, 4-D (1.1kg ha^{-1}), before planting, paraquat (2.2kg ha^{-1}) was applied, and immediately after planting, atrazine (1.7kg ha^{-1}) and metolachor (1.7kg ha^{-1}) were applied. Glyphosate-treated plots received 2.2kg ha^{-1} applied twice during the early and late crop seasons respectively. The early crop season application was sprayed over the top and the late crop season application was applied directly to the base of the plant. No-exposure plots received no glyphosate at any point. In plots with non-GR corn, glyphosate application was administered via hooded sprayer between corn rows to avoid killing those plants. To manage yellow nutsedge, halosufuron (0.07kg ha^{-1}) was applied in the third week of May. All plots additionally received a mixture of liquid urea and ammonium nitrate, providing them with 225kg N.

Root Collection and Processing

Using a flame-sterilized shovel, seven root balls were removed from each plot, beaten against the shovel to shake off excess dirt, and stored in a paper bag for transit (time unspecified). Once transferred to the lab, root balls from the same plot were then placed in a bucket with 4L of sterile MilliQ water one after another and agitated gently to remove soil. The root balls were washed with distilled water in a separate container to reduce soil further, at which point a portion of root material was removed and stored in Ziploc bags at -80^{0}C. Bags were labelled with designations to clarify the tillage type, pesticide treatment, and corn type each root sample represented.

Root Preparation

Frozen root samples were thawed, weighed, and 2.0g of 1-2cm cuts of root placed into a 50mL tared Falcon tube containing 25mL of Silwet buffer (composition being 5.7g NaH_2PO_4 x H_2O, 12.38g Na_2HPO_4 x $7H_2O$, and 150mL Silwet L-77 in 750mL MilliQ water). These tubes were vortexed to remove soil from roots, which were transferred to fresh Falcon tubes containing 25mL of Silwet buffer. The vortexing and transference steps were repeated an average of five times until all visible soil was dislodged from roots. Tubes were then sonicated at 40W output for 30 seconds in a Cole Parmer 4710 Series Model CP 100 Ultrasonic Homogenizer, then placed on ice for 1 minute to dislodge microbes from the root surface. The sonication and ice steps were repeated five times each. Roots were again transferred to a fresh, empty Falcon tube, while being clipped into smaller 0.5 cm lengths. The roots were left undisturbed in the Falcon

tube for 15 minutes to allow excess Silwet buffer to collect in the tube. Roots were then removed and stored in a clean, dry Falcon tube at -80^{0}C.

At all points during this procedure, roots were handled with flame-sterilized forceps, and all clipping was carried out by flame-sterilized scissors. The sonication probe was wiped after each root sample's sonication step was completed to remove contaminants, after which followed 90% ethanol sterilization.

Root Disruption

Root samples were removed from -80^{0}C and using flame-sterilized tweezers, approximately half of the root sample was immediately transferred into a clean, sterile tissue grinder. The other half of root sample was stored at -20^{0}C temporarily. The frozen root sample was ground into a uniform semi-liquid paste, which was then transferred into a 2mL labelled collection tube. The other half of the sample then was ground in the same tissue grinder to a similar consistency, then added to the same collection tube. This collection tube was then returned to the -80^{0}C freezer.

Root Cleanup

Each ground root sample was divided between 2 collection tubes (5mL) in approximately equal halves, and 3mL nuclease-free, autoclaved, 0.2μL filtered water was added to each. A flame-sterilized scoop was then used to add 6 glass beads to each tube. Tubes were then disrupted so that roots would not compact at one end of the tube. Tubes were placed on a shaker running at its maximum speed and at an angle (again, to prevent root compaction), secured with rubber bands, and allowed to shake for 2 hours. Following this, tubes were centrifuged at 300 x

g for 1 minute to separate the liquid and solid contents of each tube. The liquid contents of each tube were pipetted out and run through a 2µL filter into 2 labelled, tared collection tubes. Pipette transfer was performed between tubes as needed to ensure that both tubes had 0.1g of liquid contents. The resultant collection tubes were centrifuged at 15,000 x *g* for 15 minutes, after which supernatant was immediately poured off and the pellet stored at -80^{o}C for DNA extraction.

DNA Extraction and Sequencing

Extraction was carried out following a slightly modified version of the Mo Bio Powersoil® DNA Isolation Kit instructions, as explained below. The provided solution was pipetted out of the clean, labelled PowerBead tubes and used to suspend the sample pellet before the mixture was replaced in its original PowerBead tube along with 60µL of provided Solution C1. PowerBead tubes were then secured and shaken on a flat-bed vortexer for 15 minutes. Tubes were centrifuged at 10,000 x *g* for 30 seconds and resultant supernatant was transferred to clean, labelled 2mL collection tubes. 250µL of provided Solution C2 was pipetted into these tubes. The tubes were vortexed for 5 seconds and then incubated at 4^{o}C for 5 minutes, after which they were centrifuged at 10,000 x *g* for 1 minute. Avoiding the resultant pellet, 600µL of supernatant was pipetted into clean, labelled 2mL collection tubes, to which 200µL of Solution C3 was added, vortexed for 5 seconds, and incubated at 4^{o}C for 5 minutes. The tubes were centrifuged again at 10,000 x *g* for 1 minute. Another pellet resulted and was avoided to pipet 600µL of supernatant into a clean, labelled 5mL collection tube. 1,200µL of Solution C4 was added and vortexed for 5 seconds.

At this point, the two duplicates of each sample were recombined using a Powervac ™ Manifold, passing the tubes' contents through a spin filter column in 600µL increments. 800µL of 100% ethanol was then passed through the spin filter, followed by 500µL of Solution C5, with each addition being allowed to drain completely before the stopcock was turned. The vacuum was run for an additional 1 minute to allow the spin filter membrane to dry, then turned off to remove the spin filter columns and replace them in their original 2mL collection tubes, which were then centrifuged at 13,000 x *g* for 1 minute to fully dry the membrane. The spin filter column was then transferred to a clean, labelled 2mL collection tube, where 100µL of Solution C6 was added to the center of the white filter membrane. The tubes were centrifuged at 10,000 x *g* for 30 seconds before the spin filter was discarded and the remaining liquid was transferred to a -80^{0}C freezer until it could be primed and transferred for PCR amplification and subsequent 16S rRNA sequencing. The ultimate product was 33 samples, each combination of treatments replicated 4 times for a total of 32 samples, plus a control where all steps were carried out as written but without any actual root tissue (so as to correct for potential procedural contamination and human error).

Illumina MiSeq 16S rRNA Mothur Sequence Analysis

The initial data from the sequencing process provided four replications per sample. Six samples returned insufficient sequence data and were discarded. The discarded samples were the procedural contamination control, as well as two reduced tillage/no-glyphosate/GR samples, one conventional tillage/glyphosate/GR sample, one conventional tillage/glyphosate/non-GR sample, and one conventional tillage/no-glyphosate/non-GR sample.

All four replicate sequencing runs for each sample were treated as a single sample during mothur processing. Mothur is a free, Windows-compatible software intended to simplify 16s rRNA sequence analysis [13]. The replicates were organized in a .files file which assigned the appropriate MiSeq .fastq reads to each sample. Table 1 shows the order of mothur instructions used to provide diversity analyses, sequence screening, and sequence alignment, among other features [13] used to generate the experiment's data.

Table 1. Commands used in the bioinformatics software mothur to process the 16S rRNA gene sequence data obtained in this study

Command	Purpose
make.contigs	Reads were merged into a .fasta file of all the sequences as well as a .groups file keeping track of which sequences came from which sample.
summary.seqs	Provided a table showing basic information about the number of sequences in the current .fasta file, the number of bases in those sequences (as a percentage), the number of ambiguous bases, and indicators of poor sequence quality. This command was used repeatedly in the procedure to be certain the commands were executing correctly.
screen.seqs(maxambig=1, maxlength=275)	To eliminate errors from sequences far exceeding the normal 250 base length, sequence size was limited to 275 base pairs, with 1 ambiguous base permitted.
unique.seqs	Generated a .names file to merge duplicate sequences and lower processing demands.
count.seqs	Generated a count table from the .names and .groups files.
align.seqs(reference=silva.v4.fasta)	Aligned the sequences using the SILVA V4 database. The SILVA database included Archaea, Eukaryota, and Bacteria domains and large and small subunit rRNA genes. It further included taxonomic classifications, multiple sequence alignment, type strain information, the latest valid nomenclature, and quality checking for all sequences [10].
screen.seqs(start=1968, end=11550, maxhomop=8)	Eliminated erroneous sequences with more than 8 identical bases in a row and mis-amplifications that did not correspond to the V4 region under examination.
filter.seqs(vertical=T, trump=.)	Non-informative gap sequences were filtered out, typically reducing the number of sequences under scrutiny.
unique.seqs	As the editing might have caused more sequences to become identical, these were eliminated.
pre.cluster(diffs=2)	Sequences so slightly different (2 bases out of 250) that they were liable to be PCR or sequencing error instead of genetic variation were pooled back together.
chimera.uchime(dereplicate=t)	Listed the chimera sequences brought about by PCR error into the .accnos file via the UCHIME procedure. The dereplicate=t subcommand caused mothur to examine each sample individually.

remove.seqs	Eliminated the listed chimera sequences.
classify.seqs(taxonomy=greengenes.tax, cutoff=80)	Using Greengenes (stored in the greengenes.fasta file), the sequences in the given files were classified and placed into Excel-compatible .taxonomy and .tax.summary files.
remove.lineage(taxon= Chloroplast-mitochondria-unknown-Archaea-Eukaryota)	Unwanted sequences such as chloroplast or mitochondrial genetic information were removed.
cluster.split(splitmethod=classify, taxlevel=4, cutoff=0.15, processors=4)	To reduce the processing time, sequences were grouped via taxonomy, and from there further clustered into operational taxonomic units (OTUs).
make.shared(label=0.03)	Created an Excel-compatible .shared file which contained how frequently a sequence belonging to a particular OTU was found in a sample.
count.groups	Checked how many sequences were in each sample so as to eliminate samples that had very low sequence counts from further analysis.
classify.otu	Identified the established OTUs and generated Excel-compatible .taxonomy and .tax.summary files.
summary.single(subsample=t, iters=1000, calc=sobs-nseqs-coverage-invsimpsom-chao-shannon-ace)	Measured alpha diversity by subsampling from each group 1,000 times. Provided the number of OTUs, number of subsampled sequences, sampling thoroughness calculations, inverse Simpson index, Schao index, Shannon index, and SACE index.
dist.shared(subsample=t)	Created a similarity matrix for samples, based on presence and abundance of OTUs.
nmds	Used non-metric multidimensional scaling to visualize how similar samples were based on the presence or absence of OTUs (jclass) and in terms of abundance of OTUs (thetayc).
corr.axes(method=spearman)	Using Spearman's rank correlation coefficient, axis scores from the NMDS ordinations were correlated with the abundance of different OTUs in an Excel-compatible file.
amova*	Examined whether the differences between treatments was greater than the differences within treatments.
indicator*	Tested for the presence of indicator OTUs to show the different distribution of OTUs between sample groups.

*These commands required "design" files which were set up to compare the different sample groups. Three separate design.files distinguished between samples with conventional and reduced tillage, GR and non-GR plants, and glyphosate and no-glyphosate treatments in order to allow mothur to compare sample compositions. In a fourth file, total variable combinations were compared to one another.

RESULTS AND DISCUSSION

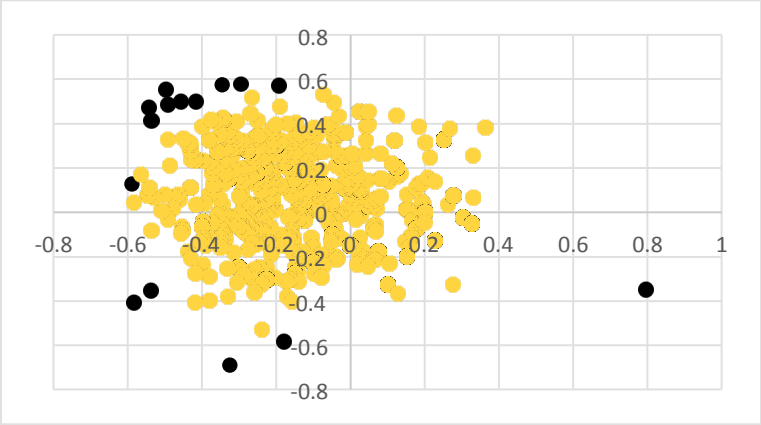
Alpha diversity analyses looked into the richness of community composition in the individual samples [15] and these results were used to generate the beta diversity analyses. Data

was primarily analyzed via three different beta diversity analyses, each of which compared the samples' distinct microbial compositions for significant differences.

Spearman's Correlation Coefficient and NMDS Analysis

Non-metric multidimensional scaling (NMDS) was used to represent how similar samples were to each other. This generated a graph of which bacterial taxa appeared responsible for the greatest differentiation between samples. In Figure 1, the most prominent OTUs are highlighted in black while the other bacteria are denoted in yellow.

Figure 1. NMDS plot of the bacteria that appeared most responsible for the greatest differentiation between samples.



The axes from Figure 1 were correlated with OTU abundance via Spearman's rank correlation coefficient. The distance from the NMDS graph's origin point is the value presented as length in Table 2. All length values calculated to be above 0.75 were considered significant and the fifteen OTUs indicated to have the most significance were identified in Table 2 according to this length value.

Table 2. The fifteen most significant OTUs, as calculated by NMDS, OTU abundance, and the Spearman's rank correlation coefficient.

OTU	Identity	Length
OTU 0001	*Escherichia coli*	0.86907
OTU 0004	*Acinetobacter guillouiae*	0.648784
OTU 0005	unclassified *Burkholderia*	0.762337
OTU 0006	unclassified *Acidovorax*	0.649012
OTU 0009	unclassified *Cloacibacterium*	0.601773
OTU 0010	*Rhizobium leguminosarum*	0.710105
OTU 0037	unclassified *Pseudomonadaceae*[f]	0.743236
OTU 0076	*Bosea genosp.*	0.609317
OTU 0092	unclassified *Sphingomonas*	0.602735
OTU 0099	unclassified *Aeromonadaceae*[f]	0.677028
OTU 0136	unclassified *Prevotella*	0.671877
OTU 0164	*Arthrobacter psychrolactophilus*	0.719996
OTU 0185	unclassified *Actinomycetales*[o]	0.677416
OTU 0262	*Dongia mobilis*	0.643942
OTU 0620	unclassified *Koribacteraceae*[f]	0.69042

[f] indicates classification continued only to the family level

[o] indicates classification continued only to the order level

The results indicated OTUs 0001 and 0005 were the only two sequences with length sufficient to be considered significant, suggesting only limited differences between sample compositions. Meanwhile, OTUs 0006 and 0185 were both listed as being significant indicators of difference in later beta diversity analyses, which potentially supports their being a relatively large presence despite not meeting the significance requirements for this particular analysis. OTU 0001 was also the largest presence among the samples, its OTU designation of 0001 indicating that this sequence was seen in aggregate more times than any other OTU (OTU frequency increases inversely to OTU numeric designation).

AMOVA Analysis

Exploration of the differences between samples continued with analysis of molecular variance (AMOVA; Table 3).

Table 3. Differences between treatments as determined from AMOVA analysis. Variables are abbreviated as glyphosate treatments (GLY), no glyphosate (nGLY), reduced tillage (RT), conventional tillage (CT), GR (same), and non-GR (nGR).

Sample Groups Compared	p-value
All possible combinations	<0.001*
CT and RT	0.015*
nGR and GR	0.031*
CT/nGLY/nGR and CT/nGLY/GR	0.037
CT/nGLY/GR and RT/nGLY/nGR	0.063
CT/nGLY/GR and CT/GLY/nGR	0.076
CT/nGLY/nGR and CT/GLY/GR	0.087
CT/GLY/GR and RT/noGLY/nGR	0.088
CT/nGLY/nGR and RT/nGLY/nGR	0.094
CT/GLY/nGR and CT/GLY/GR	0.096
CT/GLY/nGR and RT/GLY/nGR	0.099
CT/nGLY/nGR and CT/GLY/nGR	0.1
CT/GLY/nGR and RT/nGLY/nGR	0.102
CT/GLY/GR and RT/nGLY/GR	0.104
CT/nGLY/nGR and RT/GLY/GR	0.105
CT/nGLY/nGR and RT/GLY/nGR	0.107
CT/GLY/GR and RT/GLY/GR	0.115
CT/nGLY/GR and RT/nGLY/GR	0.128
CT/GLY/nGR and RT/nGLY/GR	0.199
CT/GLY/GR and RT/GLY/nGR	0.208
CT/nGLY/nGR and RT/nGLY/GR	0.208
RT/nGLY/nGR and RT/nGLY/GR	0.216
RT/nGLY/nGR and RT/GLY/GR	0.295
CT/nGLY/GR and CT/GLY/GR	0.341
RT/nGLY/GR and RT/GLY/GR	0.364

CT/GLY/nGR and RT/GLY/GR	0.388
CT/nGLY/GR and RT/GLY/GR	0.449
RT/nGLY/nGR and RT/GLY/nGR	0.45
RT/nGLY/GR and RT/GLY/nGR	0.571
CT/nGLY/GR and RT/GLY/nGR	0.58
GLY and nGLY	0.69
RT/GLY/nGR and RT/GLY/GR	0.844

*indicates p-value was considered significant in AMOVA

A simultaneous comparison of all eight combinations of variables led to $p<0.001$, which was significant; therefore the differences between all eight sample groups exceeded the variation within the samples themselves. This indicated that, as a group, the variables studied here (tillage, glyphosate application, and transgenic corn type) did have an effect on the bacterial endophyte community composition. However, when sample treatments were compared individually to one another, AMOVA was unable to significantly detect differences between any individual pairs of treatments. When variables were strictly compared only to their counterpart, AMOVA was also unable to detect significant differences between GLY and nGLY. It did detect significant differences between GR and nGR corn plants, ($p = 0.031$), and between RT and CT ($p = 0.015$). This indicated that tillage approach and genetically modified plants (compared to non-modified) both led to a significant difference in the endophyte community.

Indicator OTU Analysis

Sequences were tested for indicator OTUs (those which differed significantly between samples; Table 4).

Table 4. Indicator OTUs displayed with their identities as far as the greengenes database could classify their sequences, the samples they were predominant within, and p-values for the significance of these differences.

OTU	Identification	Presence	P-value
0006	unclassified *Acidovorax*	CT/nGLY/nGR	0.047
0029	unclassified *Kaistobacter*	RT	0.003
0029	unclassified *Kaistobacter*	RT/nGLY/GR	0.044
0035	unclassified *Sinobacteraceae*[f]	RT/nGLY/GR	0.043
0036	*Lechevalieria aerocolonigenes*	GR	0.039
0048	*Prevotella melaninogenica*	CT/nGLY/nGR	0.048
0055	*Rothia mucilaginosa*	CT/nGLY/nGR	0.044
0063	unclassified *Kaistobacter*	GR	0.035
0064	*Sphingomonas wittichii*	RT	0.025
0065	unclassified *Chryseobacterium*	nGLY	0.044
0065	unclassified *Chryseobacterium*	CT/nGLY/nGR	0.024
0080	unclassified *Prevotella*	CT/nGLY/nGR	0.045
0088	unclassified *Alicyclobacillus*	GR	0.037
0096	unclassified *Gaiellaceae*[f]	RT/nGLY/GR	0.024
0102	*Streptomyces reticuliscabiei*	GR	0.042
0139	unclassified *Actinomycetales*[o]	RT/nGLY/GR	0.013
0144	unclassified *Solibacillus*	CT/nGLY/nGR	0.038
0169	unclassified *Lachnospiraceae*[f]	CT/nGLY/nGR	0.048
0180	unclassified *Clostridium*	CT/nGLY/nGR	0.046
0183	unclassified *Candidatus Xiphinematobacter*	RT/nGLY/GR	0.007
0185	unclassified *Actinomycetales*[o]	CT/nGLY/nGR	0.019
0190	unclassified *Prevotella*	nGR	0.036
0209	unclassified *Intrasporangiaceae*[f]	RT	0.047
0216	unclassified *Streptococcus*	CT/nGLY/nGR	0.049
0289	unclassified *Actinomycetales*[o]	RT/nGLY/GR	0.012
0385	unclassified *Neisseriaceae*[f]	CT/nGLY/nGR	0.048
0442	unclassified *Chitinophagaceae*[f]	RT/nGLY/GR	0.017
0475	unclassified *iii1-15*[o]	RT/nGLY/GR	0.01
0480	unclassified *Catellatospora*	RT/nGLY/GR	0.011
0726	unclassified *Chitinophagaceae*[f]	RT/nGLY/GR	0.012
0739	unclassified *Ruminococcus*	CT/nGLY/nGR	0.047
0774	unclassified *Gammaproteobacteria*	RT/nGLY/GR	0.01
0875	unclassified *Coprococcus*	CT/GLY/nGR	0.043
0882	unclassified *Gaiellaceae*[f]	RT/nGLY/GR	0.01

[f] means classified only to family level

[o] means classified only to the order level

The only significant difference between the GLY and nGLY treatments was OTU 0065 (unclassified *Chryseobacterium*), which was found to be more prevalent in nGLY samples. This same OTU was also present in CT/nGLY/nGR samples (a specific subtype of nGLY sample). As none of the other OTUs from that variable combination were noted during this particular comparison, it can be assumed that either OTU 0065 was particularly prevalent in other nGLY samples compared to the other OTUs in CT/nGLY/nGR, or the other significant OTUs from CT/nGLY/nGR were sufficiently present in GLY samples to conceal their prevalence in nGLY samples. It is important to note that the RT/nGLY/GR sample mentioned in the methods may have affected the GLY/nGLY comparison results, though this effect would be mitigated by many other GLY and nGLY samples.

When the samples were divided by tillage treatment, OTUs 0029 (unclassified *Kaistobacter*), 0064 (*Sphingomonas wittichii*), and 0209 (unclassified *Intrasporangiaceae*) were significantly present in RT samples; no OTUs were significantly more prevalent in the CT samples. OTU 0029 (and none of the others) was also represented as significant in a specific variable combination group (RT/nGLY/GR), indicating particularly high levels within that group. This group was mentioned before as returning two samples with no data, and these results are accordingly inconclusive. Additionally, OTU 0209's significance may be the overemphasis of a rare bacterium due to a comparatively rare presence in the data set. Overall, however, the presence of multiple significantly different OTUs between CT and RT samples supports the AMOVA suggestion of significant difference due to tillage treatments; these OTUs can be assumed to be what that difference was derived from.

In terms of GR and nGR corn, OTUs 0036 (*Lechevalieria aerocolonigenes*), 0063 (unclassified *Kaistobacter*), 0088 (unclassified *Alicyclobacillus*), 0102 (*Streptomyces reticuliscabiei*), and 0190 (unclassified *Prevotella*) all differed significantly. All but OTU 0190 were present predominantly in GR corn. Overall, the presence of these OTUs again reflects the AMOVA findings. The risk of slightly biased data from the RT/nGLY/GR sample is likely compensated for by the many other GR and nGR samples. Of some concern is the significance of OTU 190, an unidentified *Prevotella*, a genus of gram-negative, obligately anaerobic, nonmotile, nonsporeforming, pleomorphic rods, that contains several species found in the oral cavity [16]; accordingly, it may have resulted from contamination. Both it and OTU 102's relatively smaller presence in the samples may suggest that they were the product of overemphasis.

When samples were compared by variable combinations, two combinations were revealed to be especially unique. The first was CT/nGLY/nGR samples, which showed OTUs 0006 (unclassified *Acidovorax*), 0048 (*Prevotella melaninogenica*), 0055 (*Rothia mucilaginosa*), 0065 (unclassified *Chryseobacterium*), 0080 (unclassified *Prevotella*), 0144 (unclassified *Solibacillus*), 0169 (unclassified *Lachnospiraceae*), 0180 (unclassified *Clostridium*), 0185 (unclassified *Actinomycetales*), 0216 (unclassified *Streptococcus*), 0385 (unclassified *Neisseriaceae*), and 0739 (unclassified *Ruminococcus*) to be present significantly in comparison to the rest of the endophyte samples. OTU 0006 and 0185 both were distinguished in the NMDS analysis as having potentially significant presences in the data set. No other OTUs from the NMDS analysis were seen in the indicator OTUs analysis results, lending these two a special significance and accordingly making the CT/nGLY/nGR variable combination appear to have a drastic effect on endophyte microbial community composition. OTU 0169's *Lachnospiraceae*

family typically contains gastrointestinal bacteria in humans and ruminants, all of which are strict anaerobes and primarily nonsporeforming. Its members can also occur in the human oral cavity [8] and its presence may indicate contamination. Likewise, OTU 0055, *Rothia mucilaginosa,* is an upper respiratory bacteria that causes opportunistic infections in severely immunocompromised human hosts [7], and almost certainly resulted from contamination, but the other prokaryotes have more ambiguous origins. OTU 0065 was mentioned in previous comparisons (nGLY and GLY), which may suggest that the CT/nGLY/nGR combination was affected by that comparison or vice versa. Any of the OTUs 0144, 0169, 0180, 0185, 0216, 0385, and 0739 could be rare, overemphasized bacteria. Conversely, it is possible that the combinations of one variable in conjunction with another may have had some holistic effect upon the endophyte composition, affecting OTU 0065 as well as others (especially OTU 0006 and 0185 from the NMDS analysis).

The second significant variable combination referred to RT/nGLY/GR samples, which, due to low returns, only had two samples of the four intended for this experiment. Accordingly any results are unreliable and are less likely to be indicative of actual differences in the endophyte community composition than other comparisons. OTUs 0029 (unclassified *Kaistobacter*), 0035 (unclassified *Sinobacteraceae*), 0096 (unclassified *Gaiellaceae*), 0139 (unclassified *Actinomycetales*), 0183 (unclassified *Candidatus Xiphinematobacter*), 0289 (unclassified *Actinomycetales*), 0442 (unclassified *Chitinophagaceae*), 0475 (unclassified *iii1-15*), 0480 (unclassified *Catellatospora*), 0726 (unclassified *Chitinophagaceae*), 0774 (unclassified *Gammaproteobacteria*), and 0882 (unclassified *Gaiellaceae*) were significantly in this group. As with the CT/nGLY/nGR sample above, none of these OTUs except for 0029 were seen in the previous analyses, indicating either overemphasis of rare microbes or a potential

holistic effect of these combined variables. In the case of this variable combination, more study would be required to confirm or deny these suppositions, but as only three of its OTUs have designations less than one hundred, it is likely that several of the OTUs described were not so significant as they appeared.

A third variable combination (CT/GLY/nGR) had OTU 0875 (unclassified *Coprococcus*) represented to a significantly heightened degree. This may reflect another holistic effect of the variable combinations, albeit a much more subtle one, or the previously discussed overemphasis of rare bacteria, which is more likely as the OTU designation of 0875 indicates the bacteria to be present in relatively low numbers.

CONCLUSION

Although the prevalence of *E.coli* indicates that there was likely contamination, its lack of appearance as a significant OTU in any of the sample groups suggests that the contamination in question was reasonably uniform, and thus cannot invalidate the other results. The combination of NMDS, AMOVA, and OTU indicator testing indicate that tillage treatments and GMO corn plants both likely influence prokaryotic endophyte community composition. Whether or not glyphosate treatments have an effect is less certain, and if so, it appears to be a rather subtle one (at least within the time frame of this experiment). There also was a suggestion of variables combining to have net effects on the endophyte community composition, particularly when dealing with CT/nGLY/GR (and also possibly with RT/nGLY/GR, though the unreliability of the latter combination makes it impossible to say within the scope of this experiment). These results suggest that to understand the full nature of endophyte communities, it will not be enough

to simply select straightforward variables; combinations of variables may affect each other to a significant degree. There is also potential to discover unanticipated effects of these variables on the endophyte community composition over periods of time longer than one year, especially given climatological variations and the potential consequences therein.

Many of the OTUs considered significant were unable to be identified by the current databases. As so many of these prokaryotes remain unknown, it is difficult to speculate as to the broader effects of any of the variables examined in this experiment on the agricultural process or the ecological ramifications. It may prove possible to derive some understanding from examining phylogenetic data, especially given the improvements in accurate prokaryote classification over the recent years and optimally, the field of microbiology will only continue to grow. The continuing expansion of the databases will allow future experiments with endophytes to yield more telling results.

Larger sample sizes could also be used in the future, to minimize the risk of procedural error and the consequences of data loss. Though it is currently beyond the scope of this experiment, future studies could compare the endophyte communities of plants other than *Zea mays*, and hopefully such studies will pave the way to a broader understanding of prokaryotes, their role in our ecosystem as a whole, and the application of the latest agricultural techniques. Finally, to address the issue of contamination, future endophyte research should continue to modify the experimental protocol at any given level in order to better isolate the desired bacterial endophyte rRNA and reduce contamination.

BIBLIOGRAPHY

1. **Boddey, R. M., Urquiaga, S., Reis, V., and Dobereiner, J**. 1991. Biological nitrogen fixation associated with sugar cane. Plant & Soil. **137**: 111-117.
2. **Caporaso, G. J., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M. et al**. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal. **6**: 1621-1624.
3. **Carpenter-Boggs, L., Stahl, P. D., Lindstrom, M. J., and Schumacher, T. E.** 2003. Soil microbial properties under permanent grass, conventional tillage, and no-till management in South Dakota. Soil & Tillage Research. **71**: 15-23.
4. **Duke, S. O. and Powles, S. B**. 2008. Glyphosate: a once-in-a-century herbicide. Pest Management Science. **64**: 319-325.
5. **Groenigen, K., Bloem, J., Baath, E., Boeckx, P., Rousk, J., Bodé, S., Forristal, D., and Jones, M. B**. 2010. Abundance, production and stabilization of microbial biomass under conventional and reduced tillage. Soil Biology & Biochemistry. **42**: 48-55.

6. **Hirsch, G. and Braun, U.** 1992. Communities of parasitic microfungi. In: W. Winterhoff editors. Handbook of Vegetation Science. Vol 19, Fungi in Vegetation Science. Kluwer Academic Dordrecht, p. 225-250.

7. **Lee, A. B., Harker-Murray, P., Ferrieri, P., Schleiss, M. R., and Tolar, J**. 2007. Bacterial meningitis from *rothia mucilaginosa* in patients with malignancy or undergoing hematopoietic stem cell transplantation. Pediatric Blood & Cancer. **50**(3): 673-676.
8. **Meehan, C. J. and Beiko, R. G**. A phylogenetic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. 2014. Genome Biology and Evolution. **6**(3): 703-713.
9. **Padgette, S.R., Kolacz, K. H., Delannay, X., Re, D. B., LaVallee, B. J., Tinius, C. N., Rhodes, W. K., Otero, Y. I., Barry, G. F., Eichholtz, D. A. et al.** 1995. Development, Identification, and Characterization of a Glyphosate-Tolerant Soybean Line. Crop Science. **35**(5): 1451-1459
10. **Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O**. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research. **41**: D590-D596.
11. **Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., and Seralini, G.** 2005. Differential Effects of Glyphosate and Roundup on Human Placental Cells and Aromatase. Environmental Health Perspectives. **113**(6): 716-720.
12. **Schloss, P. D.** 2010. The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. PLOS Computational Biology. **6**(7): e1000844. doi: 10.1371/journal.pcbi.1000844.

13. **Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J. et al**. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology. **75**(23): 7537-7541.

14. **Schmidt, T. M., DeLong, E. F., and Pace N. R**. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. Journal of Bacteriology. **173**(14): 4371-4378.

15. **Sepkoski, J. J**. 1988. Alpha, beta, or gamma: where does all the diversity go? Paleobiology. **14**(3): 221-234.

16. **Shah, H. N. and Collins, D. M.** 1990. Prevotella, a new genus to include *bacteroides melaninogenicus* and related species formerly classified in the genus bacteroides. International Journal of Systemic Bacteriology. **40**(2): 205-208.

17. **Stone, J. K., Bacon, C. W., and White, J. F., Jr**. 2000. An overview of endophytic microbes: endophytism defined. In Bacon, Charles W. and White, James F., Jr. editors. Microbial Endophytes. Marcel Dekker, New York, p. 3-20.

18. **Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tse, H., and Yuen, K.Y**. 2008. Then and now: use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology and Infection. **14**(10): 908-934.